Prevalence of Shiga-toxin-producing *Escherichia coli* from milk samples of some cattle farms Al-Buḥayrah Governorate Egypt

Abstract

Objective:*Escherichia coli* (*E. coli*) contamination of milk can pose significant public health and economic concerns. The current study was conducted to explore the prevalenceand antibiotic resistance profiles of Shiga-toxin-producing *Escherichia coli* in milk samples of dairy cows in Egypt.

Study design: Twenty milk specimens were gathered from dairy cattle (ten from healthy cows and ten from mastitic cows) and examined for the presence of Shiga-toxin-producing *E. coli* using selective culture media. Molecular detection of virulence genes and phenotypic antimicrobial resistance were performed.

Results:*E. coli* was isolated from 65% (13/20) of the total milk samples (90% from mastitic cows and 40% from normal healthy cows). With 84.62% (11/13) for*Stx*2a, 69.23% (9/13)for *Stx*2f, and 61.54% (8/13)for *Stx*1 and *Stx*2 being the most predominant. Antibiotic resistance was observed againstAmikacin, Ciprofloxacin, Erythromycin, Linezolid, Penicillin G, and Trimethoprim/Sulfamethoxazole with percentages of 100%, Amoxicillin/ Clavulanic acid, and Tylosin, was noticed at 92.3% and Oxytetracycline resistance was observed at 84.62% of isolates indicating multidrug-resistant profiles.

Conclusions: The presentwork highlights the presence of various *E. coli* strains, including potential pathogens and multidrug-resistant isolates, in milk specimens from dairy cows. Milk testing regularly and improved mastitis control measures arethe key to reduce the ~~ing~~ public health risks and to safeguard~~eing~~ dairy farm productivity.

Keywords:*E. coli*, milk, dairy cows, virulence genes, antibiotic resistance~~,~~

1. Introduction

Milk is regarded as the nature's most complete food. However, ~~Milk is an~~ it could also act as an idealcause of severe threats and spreadsa wide varietyof harmful micro-organisms to humans, such as pathogenic *Escherichia coli* (*E. coli*)**[1,2]**.Bovine mastitis is one among the most prevalent and ~~s among the most public and~~ costly diseases in dairy cows worldwide **[3,4]**.In particular,*E. coli* is one of the most prevalent and ~~potential~~potent pathogen~~s~~ that causes mastitis in dairy cattle**[5,6]**. The organism is a common inhabitant of the ruminant's gastrointestinal tract which can

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fo hcae tnieb ot retteB **:[8D]Comment** rettel latipac a htiw drowyek eht invade and damage the mammary gland tissues through the teat canal **[7]**.Isolation of the pathogenic *E. coli* strainshas been reported from raw milk samples ~~lings~~**[8,9,10]** and also from bovine mastitic milksamples **[11,12,13]**in Egypt and all over the world.Enteropathogenic *E. coli*, enterotoxigenic *E. coli*,and enterohemorrhagic *E. coli* which are known as lethal Shiga toxin *or* verotoxin-producing *E. coli* strains are categorized based on theirvirulence factors**[14,15,16]**. STEC strains are a significant group of bovine mastitis pathogens, ~~for bovine mastitis~~ as recorded in several studies **[11,17]**. Among the most important virulence genes were ~~S~~higa toxins (Stx1, Stx2) and *eae*(intimin) in *E. coli* strains isolated from milk samples ~~lings~~ of bovine mastitis **[18,19]**.

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In the last few decades, antimicrobial resistance (AMR) has become a significant threat to public health around the world **[20]**. The ~~In Veterinary Medicine, the~~ hysterical and excessive~~mis~~use of antibioticsin dairy cattleraises the AMR incidence in mastitis pathogens ~~raw milk~~**[21]**.~~Some P~~previous studies ~~in the past~~ have indicated ~~in the past~~ that there was no AMR among mastitis pathogens ~~the causes of bovine mastitis~~**[22,23]**. However, ~~Despite that,~~ in recent years, pathogens such as *E. coli* that are resistant to several antibacterials have been discovered, which may be spread from cattle to humans through milk. **[24,25]**. AMR investigation studies have selected *E. coli* as a sentinel pathogen in investigation studies related to antibiotic resistance because of its ease~~y~~ acquisition of resistance and its presence in the intestinal tract of humans and animals **[26]**.Consequently, this work aimed to study the prevalence of *E. coli* in milk samples from mastitic and normal dairy cattle in Egypt. Additionally, the determination of their virulence factors and antibacterialsensitivity tests for ~~the isolates~~ were achieved to provide vital consideration for the management of the dairy sector in Egypt.

2. Materials and methods

2.1. Sample collection ~~ings~~

This study was performed during the period from January 2019 to July 2020. A total of twentymilksamples were assembled from Egyptian dairy cattle. Ten samples were from mastitic cows and ten samples were from healthy cows.These samples were collected during hand milking inthe early morning (6.00-7.00 am) under aseptic conditions ~~in complete hygienic measures~~. Fresh milk samples (about 100 ml) were placed ina sterile milk collection vials ~~bottle. These bottles were kept in an ice box at 4°C after labeling. Then~~ and transported to the Department of Bacteriology, Mycology, and Immunology laboratory, Faculty of Veterinary Medicine, Sadat City University for examination in the ~~Department of Bacteriology, Mycology, and Immunology laboratory.~~

2.2. Isolation and identification of *E. coli*

Pre-enrichment in nutrient broth and incubation for 24 hrs at 37°C was performed ~~for the milk samplings~~ shortly after arrivaland the milksamples was initially inoculated on to ~~.An incubation period of 24 hours was conducted after a loopful of the culture appeared to be growing on~~ MacConkey Agar ~~r medium plates~~ and incubated at~~at~~ 37°C.A sterile loop was used to transfer ~~pick up t~~the pink lactose fermenting colonies ~~and transferred them~~ to Eosin Methylene Blue agar ~~for 24 hours at 37°C. The semisolid medium was inoculated with isolated E. coli colonies with a~~ which produced the characteristic bluish green colonies with a metallic green sheen. ~~, and the inoculum was incubated for 24 hours at 37°C and maintained at 4°C.~~ Additional morphological, biochemical, and molecular characterization was done on the suspicious colonies.Different biochemical tests, like cytochrome oxidase, indole test, triple sugar iron agar, and urease, were used to confirm the isolated E. coli strains at the species level**[27,28]**.

2.3. ~~The~~Characterisation of virulence genes ~~of *E. coli*serotypes characterization~~

E. coli serotypes were inoculated on MacConkey agar medium plates after it had been preserved on semisolid medium. Following an overnight incubation period at 37°C, a small number of colonies were chosen using a sterile toothpick in order to extract DNA using the QI Aamp Miniprep kit, following the manufacturer's recommendations. Primers that target *stx*1, *stx*2, *eae*A, and *hly*A were used to check for the presence of virulence genes in all of the obtained *E. coli*serotypes.

2.4. Antimicrobial susceptibility test

Using 0.5 McFarland tube ~~0.5~~, all confirmed isolates were cultured on Mueller-Hinton (Oxoid) broth and incubated at37°Cfor a period of 18 hours at ~~37°C~~, till the density of bacteria was set to 1.5x10^8/~~1~~ ml. Following that, Mueller Hinton agar (Oxoid) plates were covered with 1 ml from each tube. The disk diffusion technique was used to determine susceptibility to 21 antimicrobials based on the Clinical and Laboratory Standards Instituteguidelines**[29]**. The tested antibiotics wereAmikacin 30µg,Amoxicillin/Clavulanic acid30µg, Ampicillin 10µg,Ceftiofur 30µg,

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Ceftriaxone30µg,Chloramphenicol15µg,Ciprofloxacin, Doxycycline20µg, Gentamicin, linezolid 30µg, marbofloxacin 5µg,Nalidixic acid30µg, Nitrofurantoin200µg,Oxytetracycline20µg,Penicillin G 10µg, Streptomycin5µg,Trimethoprim/Sulfamethoxazole25µg, Tulathromycin 30µg, and Tylosin 30µg.

2.5. Ethical Approval

The current work was revised and approved by the Animal Use Ethics Committee of the Faculty of Veterinary Medicine, Sadat City University.

2.6. Statistical analysis

The STEC rates of isolation, the distribution patterns of virulence genes~~,~~ and the susceptibility and resistance of isolates to antimicrobials are denoted as percentage~~s~~ (%). Using the Fisher's exact test and the Z-test in R statistical software, the significance of the differences in STEC isolation rates from various sources, serotypes, antimicrobial efficacy~~,~~ and virulence gene distribution patterns was assessed at a statistical significance of p ~~P~~<0.05.

3.Results

3.1. Isolation of E. coli from milk samples ~~I~~ncidence of *E. coli*in the examined milk samplings

The results of *E. coli* isolation from twenty milk samples (ten samples from mastitis cows and ten from healthy cows) were illustrated in Table 1. Out of the twenty samples, thirteen samples with a total percentage of 65% were positive for *E. coli*~~incidence~~ (nine from mastitic milk with a percentage of 90% and four from normal and healthy milk with a percentage of 40%).

3.2. Percentage of virulence genes among the obtained isolates

The results of virulence gene profiling ~~s among~~*E. coli* isolates are presented in Table 2.The gene s~~S~~*tx2*awas detected in 11/13 (84.62%),genes~~S~~*tx2*fwas represented in9/13 (69.23%), geness~~S~~tx1 and s~~S~~*tx*2 were detected in8/13 (61.54%), gene *eae*was detected in7/13 (53.85%), genes s~~S~~*tx*1d and s~~S~~tx2g were represented in 4/13 (30.77%). While the virulence genes~~S~~*stx1*c, s~~S~~*tx*2c,s~~S~~*tx*2d,s~~S~~*tx*2e, and*ehx*Awere not detected in any of the milk samples 0/13 (0%).

3.3.Antimicrobial ~~Prevalence of antimicrobial~~resistance pattern of *E. coli* isolates

The positive *E. coli*isolates milk samples were examined for antibiotic resistance and were applied against the following antibiotics disks,ie., Amikacin,Amoxicillin/clavulanic acid, Ampicillin,Ceftiofur, Ceftriaxone,Chloramphenicol,Ciprofloxacin, Doxycycline, Gentamycin, Linezolid, Marbofloxacin,Nalidixic acid, Nitrofurantoin,Oxytetracycline,Penicillin, Streptomycin,Trimethoprim/Sulfamethoxazole, tulathithromycin, and tylosin (Table 3). The current study detected complete resistance to Amikacin, Ciprofloxacin, Linezolid, Penicillin G, and Trimethoprim/Sulfamethoxazole (100%). Moreover, resistance to Amoxicillin/ Clavulanic acid,and Tylosin were noticed at 92.3%. Oxytetracycline resistance was observed at 84.62%.On the other hand, resistance to Streptomycin (69.23%), Ceftiofur, Ceftriaxone,Chloramphenicol, and Nalidixic acid (53.85%). Whereas, the lowest percentages of resistance were recorded for ampicillin (46.15%), Nitrofurantoin (38.46%), Gentamicin and Marbofloxacin (30.77%), Doxycycline (23.08%), and Tulathromycin(15.38%). Moreover, our findings demonstrated that Tulathithromycin, Marbofloxacin, Doxycycline, and Ceftiofur were the most sensitive antibacterial agents against *E. coli* isolated s from bovine mastitis. the milk samplings.

Table 1: Isolation of *E. coli*

Samples	No. of collected samples	No. and percentage of isolates
Mastitis	**10**	**9 (90%)**
Normal milk	**10**	**4 (40%)**
Total Samples	**20**	**13 (65%)**

Table 2: Occurrence of virulence genes of *E. coli*

Isolate code	Source	Virulence factors											
		Stx$_1$	*Stx*$_{1d}$	*Stx*$_{1c}$	*Stx*$_2$	*Stx*$_{2a}$	*Stx*$_{2c}$	*Stx*$_{2d}$	*Stx*$_{2e}$	*Stx*$_{2f}$	*Stx*$_{2g}$	*eae*	*ehx*A
1	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
2	Milk	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
3	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
4	Milk	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve
5	Milk	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
6	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
7	Milk	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8	Milk	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
9	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
10	Milk	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
11	Milk	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve

12	Milk	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**-ve**
13	Milk	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**
Total		**8/13 61.54%**	**4/13 30.77%**	**0/13 0.0%**	**8/13 61.54%**	**11/13 84.62%**	**0/13 0.0%**	**0/13 0.0%**	**0/13 0.0%**	**9/13 69.23%**	**4/13 30.77%**	**7/13 53.85%**	**0/13 0.0%**

Table 3: Antimicrobial susceptibility pattern of *E. coli* isolates

No.	Amikacin	Amoxicillin/ clavulanic acid	Ampicillin	Ceftiofur 30	Ceftriaxone	Chloramphenicol	Ciprofloxacin	Doxycycline	Gentamicin	Linezolid	Marbofloxacin 5	Nalidixic acid	Nitrofurantoin	Oxytetracycline	Penicillin G	Streptomycin	Trimethoprim/sulfamethoxazole	Tulathithromycin 30	Tylosin 30
1	R	R	S	S	S	R	R	R	R	R	S	I	I	S	R	I	R	S	I
2	R	R	R	R	I	R	R	I	I	R	S	R	I	R	R	R	R	S	R
3	R	R	S	S	I	S	R	R	I	R	S	S	I	R	R	R	R	S	R
4	R	R	R	I	I	I	R	R	S	R	S	R	R	R	R	S	R	S	R
5	R	R	I	R	R	I	R	S	I	R	R	I	I	R	R	R	R	I	R
6	R	R	R	R	S	I	R	S	R	R	I	R	R	R	R	R	R	S	R
7	R	R	R	I	R	R	R	S	I	R	R	R	I	R	R	R	R	S	R
8	R	R	S	I	R	R	R	S	R	R	S	S	R	R	R	R	R	R	R
9	R	R	R	R	R	R	R	S	R	R	I	R	R	R	R	I	R	I	R
10	R	R	R	R	R	R	R	I	S	R	R	R	I	R	R	R	R	I	R
11	R	S	S	I	R	S	R	S	I	R	S	I	I	S	R	I	R	S	R
12	R	R	S	R	R	R	R	I	I	R	R	I	I	R	R	R	R	R	R
13	R	R	S	R	I	S	R	I	I	R	S	R	R	R	R	R	R	S	R
S	0/13 0%	1/13 7.7%	6/13 46.15%	2/13 15.38%	2/13 15.38%	3/13 23.08%	0/13 0%	6/13 46.15%	2/13 15.38%	0/13 0%	7/13 53.85%	2/13 15.38%	0/13 0%	2/13 15.38%	0/13 0%	1/13 7.69%	0/13 0%	8/13 61.54%	0/13 0%
R	13/13 100%	12/13 92.3%	6/13 46.15%	7/13 53.85%	7/13 53.85%	7/13 53.85%	13/13 100%	3/13 23.08%	4/13 30.77%	13/13 100%	4/13 30.77%	7/13 53.85%	5/13 38.46%	11/13 84.62%	13/13 100%	9/13 69.23%	13/13 100%	2/13 15.38%	12/13 92.3%
I	0/13 0%	0/13 0%	1/13 7.7%	4/13 30.77%	4/13 30.77%	3/13 23.08%	0/13 0%	4/13 30.77%	7/13 53.85%	0/13 0%	2/13 15.38%	4/13 30.77%	8/13 61.54%	0/13 0%	0/13 0%	3/13 23.08%	0/13 0%	3/13 23.08%	1/13 7.7%

4. Discussions

In the current study, a total of twenty milk sampl~~ings~~es (ten samples from mastitic milk and the other ten samples from ~~normal milk~~healthy cows) were investigated for the isolation of *E. coli*. The total prevalence was 65% (13/20) with a percentage of 90% (9/10) for the milk samples from mastitic cows and 40% (4/10) for the milk samples from the healthy cows.These total results of *E. coli* isolation were nearly similar (66%) toRasheed et al. **[30].** Lower results, 11%, 28.31 %, and 29.8%, from infected milk of mastitic cows were reported by Yu et al.**[31],** Majumder et al. **[32],** and Dos Santos Alves et al. **[33]** respectively.Other results showed the isolation of *E. coli* in cow's milk by a percentage of 50% **[34]**.Remarkably, several studies have analysed~~conducted~~ the incidence of *E. coli*from milk samples of mastitic dairy cattle in Egypt **[35]**and variousareas of the world, such asAlgeria **[13]**,Brazil **[36]**,China **[37]**, Ethiopia **[38,39]**, Mexico **[40]**, Turkey **[41],** and Uruguay**[42]**. These differences in the incidence of *E. coli*between the different studies could be attributed todifferent geography,farm hygiene ~~hygienic measures for cattle and the surrounding environment,~~ health status ~~conditions~~ of animals, the management practices ~~process~~, and the different laboratories that used various isolation techniques.

Concerning the obtained resultsof the virulence genes of *E. coli* isolates from milk samples, the Stx_{2a}, Stx_{2f}, Stx_1, and Stx_2, and*eae*represented by percentages of 84.62%, 69.23%, 61.54%, and 53.85% respectively. While the virulence genes Stx_{1c}, Stx_{2c}, Stx_{2d}, Stx_{2e}, and *ehx*A were not detected. These results were nearly similar to several studies that were performed in Egypt **[43,44,45,46]**. Similarly,Wang et al. identified the genes Stx_2 and *eae* in 25% of the *E. coli* isolates**[47]**. In the same line, in Switzerland,Stx_1 and Stx_2 genes were positive in samples from bovine ~~*E. coli*~~mastitis **[48]**. In contrast to our findings,Stx_1 and Stx_2 were not found in Southern Finland **[49]**. Also, genes *stx* were not identified in the *E. coli* isolates from clinical bovine mastitis in Turkey **[41]**.Additionally, the *eae* gene was negative in the study of Stephan and Kuhn**[48]**.

Regarding the findings of antibacterial resistance against *E. coli*, the isolated strains in the current study exhibited varying resistance to antibiotics. Among our results, ~~the isolate strains resistance~~ all the strains isolated were resistant to ~~was complete to~~Amikacin, Ciprofloxacin, Linezolid, Penicillin G, and Trimethoprim/Sulfamethoxazole (100%), Amoxicillin/ Clavulanic acid, and

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Tylosinwas (92.3%), Oxytetracycline (84.62%), Streptomycin (69.23%), Ceftiofur, Ceftriaxone, Chloramphenicol, and Nalidixic acid (53.85%), Ampicillin (46.15%), Nitrofuranation (38.46%), Gentamicin and Marbofloxacin (30.77%), Doxycycline (23.08%), and Tulathithromycin (15.38%). These findings exceeded that of Elsayed and his team **[34]**. Another Egyptian study recorded that the maximumisolates were resistant to highest resistances for Tetracycline, Ampicillin, Streptomycin, and Sulfamethoxazole-Trimethoprim were (27.5%), (18.9%), (18.5%), and (11.3%) respectively, while there was no resistance towards forfosfomycin and imipenem **[50]**. Moreover, Majumder et al. recorded a smaller extent of resistance tostreptomycin, tetracycline, and ampicillin by percentages of (17.7 %), (15.93 %), and (11.5 %) respectively **[32]**. Anotherstudyreported investigated that the maximumisolates were being resistant to occurrence of resistances shown against ampicillin, gentamicin, and tetracycline were (100%), (100%), and (96.87%) respectively**[9]**.The data demonstrated by Ahmadi et al. recorded that the maximumnumber of isolates were resistant antimicrobial resistance was noticed against Streptomycin, Tetracycline, and Ampicillin **[51]**. Moreover, Momtaz et al. **[11]** recorded the *E. coli* isolates resistance in mastitic milk to Penicillin, Tetracycline, andCephalothin by percentages of (100%), (57.44%), and (6.38%) respectively. In central Ethiopia, Messele et al. **[52]**stated the resistance of the *E. coli* isolates to ampicillin, sulphamethoxazole-trimethoprim, and streptomycinwere (68.7%), (50%), and (25%) respectively. Moreover, in the study performed in Punjab, India Jindal et al. **[53]**reported investigated that the61.5%) (42.3%), and (55.7%) of*E. coli* isolates were resistant ce was found for towardsOxytetracycline, Enrofloxacin, and Sulphadiazine (61.5%) (42.3%), and (55.7%) respectively. Younis et al. **[54]**reported that the antibiotic resistance of all *E. coli* isolates was nearly complete for β-lactams, Clindamycin, and Rifampin from milk samples taken from Qena, Egypt. The differences in the antibacterial resistance of the *E. coli* isolates in different areas in Egypt and different countries all over the world might be attributed to the difference in the hygienic measures and also to the misuse of veterinary antibiotic drugs in dairy bovine farms.

5- Conclusion

According to this study's results,*E. coli*remains to be one of the predominant pathogen responsible for bovine mastitis. mainreasonsfor severe economic lossesall over the world. The gene; Stx_{2a},is the most prevalent virulence gene associated with STEC followed byStx_{2f}, Stx_1, Stx_2, *eae*, Stx_{1d}, and Stx_{2g}. The majority of the isolated *E. coli* have resistance patterns to Amikacin, Ciprofloxacin, Linezolid, Penicillin G, Trimethoprim/Sulfamethoxazole, Amoxicillin/ Clavulanic acid, Tylosin, and Oxytetracycline. Moreover,Tulathithromycin, Marbofloxacin, Doxycycline, and Ceftiofurare the most effective antibacterial agents against isolated *E. coli*.Our results should inform veterinarians about the probable prognosis of *E. coli* infections in dairy cattle thereby reducing the indiscriminate usage of antibacterial agents.

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References

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